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Comparative Biochemistry and Physiology, Part B 142 (2005) 181 – 191
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Host regulation and release of parasitism-specific proteins in the system *Toxoneuron nigriceps–Heliothis virescens*

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Received 4 February 2005; received in revised form 20 June 2005; accepted 5 July 2005
Available online 27 July 2005

Abstract

The braconid wasp *Toxoneuron nigriceps* induced qualitative and quantitative changes in the protein composition of the moth *Heliothis virescens* host hemolymph. Total protein concentration was found to be higher in parasitized host 4 days after parasitism as compared to control hosts, mainly due to changes in a particular group of proteins. Host proteins with a molecular mass of 173 and 72 kDa were found in higher levels in the hemolymph of parasitized larvae as control hosts approached pupation, while an 80 kDa peptide was found in reduced concentration in the hemolymph of parasitized hosts. Levels of these three peptides were maintained throughout parasitoid development, while two of them (173 and 72 kDa) were cleared from the host hemolymph close to pupation. Besides the regulation of host proteins, three parasitism-specific proteins (PSPs) were released into the host hemolymph. Two of them (PSP1-MW=116 kDa, p*I*=6.3; PSP2-MW=114 kDa, p*I*=6.2) first appeared in the hemolymph of parasitized hosts soon after pupation of control host and increased in concentration as the parasitoid developed. The third PSP (PSP3-MW=56 kDa, p*I*=5.8) was produced towards the end of parasitoid larval development, close to parasitoid egression. Database searches based on the amino acid composition and amino terminal sequence of PSP1 and PSP2 did not produce any significant matches, while PSP3 was identified as a putative chitinase. Incubation of host derived tissues, parasitoid larvae and teratocytes in ^{35}S conditioned media suggested PSPs were a product of teratocytes. The role of the regulation of host proteins and release of PSPs by teratocytes for the successful development of *T. nigriceps* are discussed.
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Keywords: Parasitism-specific proteins; Teratocytes; Nutrition; Storage proteins

1. Introduction

Parasitoids are a very diverse group in which nutritional and physiological interactions depend on the evolutionary history of a particular host-parasitoid association (Whitfield, 1998). They have diversified strategies to subdue the host immune response, alter and regulate host metabolism, development, and synthesis of host proteins, which may greatly vary depending on the degree of interactions between host and parasitoid (ecto vs. endoparasitism; idiobiosis vs. koinobiosis). There are a number of maternally born secretions (venom, calyx fluid), larval secretions, associated symbiotic virus (PDVs), and/or secretions from teratocytes (a particular cell type derived from the developing embryo) which may all or in part be used for host development regulation (Vinson and Iwantsch, 1980). Host regulation involves, but it is not restricted to, the inhibition of the host immune defenses and regulation of the endocrine system to prepare the host as a suitable environment for parasitoid development. Developing parasitoids must compete with host tissues for nutrients, but the host is allowed to develop to a critical and nutritionally suitable stage at which time parasitoid development is exponential. Strategies vary among different groups of parasitoids according to their develop-

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1096-4959/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.cbpc.2005.07.002

mental biology, but they all lead to the accumulation or storage of host nutrients to support parasitoid larval development (Vinson et al., 2001).

The mechanisms by which parasitoids regulate many of the physiological and developmental processes of their hosts have long been thought to result in the discovery of new target systems and the development of specific molecules for insect pest control (Stoltz, 1986; Beckage and Gelman, 2004). Special attention has been drawn to virus particles symbiotically associated with certain groups of parasitoids, which are known to disrupt the host immune response, regulate the host fat body synthetic activity and control host development (see Beckage (2003)). However, successful identification and application of a parasitoid-derived protein as a new molecule for insect pest control has only been demonstrated for the teratocyte-secreted protein (TSP14) isolated from *Microplitis croceipes* (Dahlman et al., 2003; Maiti et al., 2003). Parasitoids also induce changes in the biochemical composition of the host hemolymph which could result in a significant impact on host quality and consequently, on parasitoid larval development. Besides the ability to up or down-regulated specific host proteins, parasitoids may produce proteins on their own that could be used as a nutritional resource to support parasitoid larval development (Kadono-Okuda et al., 1998; Vinson et al., 2001). Therefore, identifying the alterations in host biochemistry, the role of these biochemical processes in host regulation and parasitoid development and the isolation and characterization of the effector molecules may also improve our knowledge about the physiological and nutritional requirements necessary to sustain parasitoid development.

Toxoneuron nigriceps (Hymenoptera: Braconidae) is a larval endoparasitoid of the budworm, *Heliothis virescens* (Lepidoptera: Noctuidae). Females can successfully parasitize hosts at any larval stage from first to early fifth instar, but parasitoid development is arrested in the first instar until the host reaches the last larval stadium, at which point the parasitoid molts to the second instar (Pennacchio et al., 1993). Females of *T. nigriceps* are known to inject other fluids into the host haemocel along with the egg during the oviposition. These fluids are composed of secretions from the venom gland and calyx fluid, but they also carry virus particles associated with the parasitoids (PDV). Altogether, they disrupt the host immune system and allow the parasitoid embryo to successfully develop. PDVs and teratocytes (embryonic-derived cells) play a significant role in the control of host development. PDVs were shown to depress the synthesis of ecdysone by the host prothoracic glands, while teratocytes altered the metabolism of the circulating ecdysteroids (Pennacchio et al., 2001). Recently, it has also been shown that parasitized larvae have a higher juvenile hormone (JH) titer than control larvae, and JH metabolism could also be involved in the arrestment of host development (Li et al., 2003). However, there is strong evidence that teratocytes are the major factor involved in the arrestment of host development, since pupation was inhibited in teratocyte-injected larvae. The endocrine changes induced in the parasitized host sustain it in the larval stage, suppressing the normal physiological changes associated with the molting process. One of these changes that may have nutritional implications for the developing parasitoid is the persistence of the host storage proteins in the circulating hemolymph (Pennacchio et al., 1992, 1994). These proteins are produced during the last larval stage and are taken up by the host fat body after pupal commitment, to be used later during the metamorphic development as an energy source. Host storage proteins represent a rich source of aromatic amino acids that may be required at the end of parasitoid larval development (Vinson et al., 2001).

In the present study, we investigated whether *T. nigriceps* would produce parasitism-specific proteins and/or induce changes in the biochemistry of the host hemolymph. We found that the host protein synthesis is affected during parasitoid development and that the percent abundances of host-specific proteins, in relation to the total hemolymph protein, are regulated. We also identified three parasitism-specific proteins released by the teratocytes that appear at specific stages of parasitoid development. Amino terminal sequence of these proteins was obtained and one of them was identified as a putative chitinase, while the two remaining proteins did not have any significant matches in a database search. The role and significance of the regulation of host proteins and the synthesis and release of parasitism-specific proteins for parasitoid development are also discussed.

2. Materials and methods

2.1. Insect rearing

H. virescens (F.) were reared on an artificial diet developed by Vanderzant et al. (1962) (Corn Earworm diet, BioServe, Inc., Frenchtown, NJ, USA) and modified by the addition of propionic and phosphoric acids (Elzen et al., 1984; Syvertsen et al., 1995). *T. nigriceps* (Viereck) were reared according to Vinson et al. (1973).

2.2. Insect staging and parasitization

H. virescens larvae were maintained at controlled conditions (29+1 °C; 60%+10% r.h.; 14L:10D), and only 4th instar larvae at the head capsule slippage stage (Webb and Dahlman, 1985) were used for parasitization. Larvae were individually offered to 1–2 *T. nigriceps* females inside plastic containers (6.5 × 9.5 cm), transferred to the rearing container immediately after parasitization occurred, and kept at controlled conditions. Synchronization of larval development was obtained by selecting only those larvae that molted to the 5th instar 12 h after parasitization. Control larvae were synchronized in a similar manner.

2.3. Hemolymph sampling

Hemolymph was collected with the aid of micro-capillary tubes by severing one of the larval prolegs. Successful oviposition and parasitoid embryonic development was assessed by observing the presence of eggs or teratocytes of *T. nigriceps* in the host hemolymph. Only samples from successfully parasitized larvae were used.

Hemolymph samples used for electrophoresis were immediately collected in ice-cold TBS buffer (20 mM Tris, 0.15 M NaCl, 5 mM EDTA, pH = 7.5) containing a cocktail of protease inhibitors (1 tablet/10 mL-Complete™, Mini, EDTA-free—Boehringer Mannheim), centrifuged (1500 $g \times 2$ min) for the removal of teratocytes and/or host hemocytes, and stored at −80 °C. Samples used for protein and carbohydrate assays were collected in ice-cold 0.05% PTU in Pringle's saline, centrifuged for the removal of cellular material and stored at −80 °C.

2.4. Protein and carbohydrate assays

Total protein content of 1 μl hemolymph from parasitized and control larvae was determined using the Folin–Ciocalteu phenol reagent (Lowry Micro Method, Sigma, St. Louis, MO, USA), and the absorbance was read at 660 nm on a Beckman DU-600 spectrophotometer following the manufacturer's guidelines. Bovine serum albumin was used as a standard.

Qualitative and quantitative sugar analysis of the hemolymph of non-parasitized and parasitized larvae of *H. virescens* was performed by gas chromatography (GC). In this study, 1 μl of hemolymph in 0.05% PTU in Pringle's saline was transferred to 250 μl glass LV insert vials (Alltech Associates, Inc., Deerfield, IL, USA) and dried in a speed vacuum centrifuge under liquid nitrogen (Jouan RC 1010-Jouan Inc.). Sugars were derivatized by adding 75 μl of 1.5 mEq/mL of *N*-trimethylsilylimidazole in a silylation grade pyridine (TRI-SIL® 'Z' Derivatizing agent, Pierce Company, Rockford, IL, USA) and heat-treating at 60–70 °C for 20–25 min. After derivatization, samples were immediately injected into a Hewlett Packard 5890 capillary GC equipped with a 25 m × 0.33 mm ID vitreous silica BP1 column (Scientific Glass Engineering, Australia) (20 psi He, temperature programmed at 110 °C for 1 min, then to 310 °C at 10 °C/min), in splitless mode. Carbohydrates were identified and quantified by comparing the GC profile of the samples to that of monosaccharide (fructose, fucose, galactose, glucose, mannose, psicose, ribose, rhamnose and sorbose) and disaccharide (gentiobiose, lactose, maltose, melibiose, palatinose, raffinose, sucrose, trehalose and turanose) standards (Cônsoli and Vinson, 2002).

Five replicates were used for the experiments on the total protein and carbohydrate composition of the hemolymph from parasitized and non-parasitized *H. virescens* for each sampling time collected at 12 h intervals.

2.5. Electrophoresis

2.5.1. SDS-PAGE—protein profile

Hemolymph samples were collected every 6 h after 24 h of parasitization up to pupation of the control (108 h) and every 12 h thereafter, until parasitoid egression from the host. Samples collected in TBS were mixed 1:1 with 2× SDS sample buffer, and proteins were resolved by SDS-PAGE (Laemmli, 1970). Protein samples were heat-treated for 15 min at 65 °C, spun down and loaded on a discontinuous 5%–25% gradient acrylamide gel slab (16 cm long) (Protean II, Bio-Rad), and run at constant voltage (220 V). Samples were loaded at a concentration of 0.3 μl hemolymph/lane, in duplicates. One set was stained with Coomassie rapid blue® R-250 (GelCode, Pierce) for 1.5 h and destained overnight in water, while silver stain was used in the other set in an attempt to increase sensitivity and detect proteins at lower concentrations. Silver staining was performed according to Blum et al. (1987). Each sampling time consisted of 5 replicates for each treatment.

2.5.2. Image densitometry

Samples prepared for densitometry analyses were collected in TBS buffer every 24 h after parasitization and mixed 1:1 with sample buffer. Samples were heat-treated at 100 °C for 5 min and loaded on discontinuous 8% acrylamide gel slab (20 × 20 cm) (Bio-Rad IIxi Protean Cell). Electrophoresis was performed at 50 mA through the stacking gel and then at 70 mA through the resolving gel, until the tracking dye reached 0.5 cm from the bottom of the gel. Samples were loaded at the same concentration as described before. Gels were stained with 0.1% Coomassie blue R-250 in 1% acetic acid/40% methanol solution, and destained in multiple changes of 7% acetic acid/5% methanol solution. Gel image acquisition and quantitation were done using the Fluor-S MultiImager system and Quantity One® software (BioRad). Each sampling time consisted of 5 replicates for each treatment.

2.6. Characterization and identification of parasitism-specific proteins

2.6.1. Isoelectric point determination by IEF electrophoresis

Samples from day 4, 6, 7, 8 and 9 d after parasitization were run against hemolymph samples from control larvae collected at day 2 and 4 for determination of the isoelectric point of parasitism-specific proteins by IEF electrophoresis. Proteins were collected in TBS buffer and loaded at 40 μg / lane on a 5.0–8.0 Ready Gel IEF gel (Bio-Rad, Hercules, CA). IEF was carried out in the Mini-Protean 3 electrophoresis cell (Bio-Rad, Hercules, CA) using 7 mM phosphoric acid as the anode buffer and 20 mM lysine and 20 mM arginine as the cathode buffer. Gels were focused for 1.5 h at 200 V and then to 400 V for 1.5 h, fixed overnight in 1% trichloroacetic acid, washed, stained with Coomassie rapid blue® R-250 (GelCode, Pierce Company)

for 1 h and destained in water until desired color was obtained.

Isoelectric point of parasitism-specific proteins was confirmed after protein bands resolved from hemolymph samples of parasitized hosts were further separated by molecular weight. Selected bands cut out from IEF gels were first equilibrated in SDS buffer (0.125 M Tris–HCl-pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 65 mM dithioerythritol and ~50 mg bromophenol blue) for 15 min, and slices were loaded on a 10% SDS-PAGE slab (16 cm long) (Protean II, Bio-Rad). The gels were washed twice in distilled water and stained as described previously.

2.6.2. Electroblotting and amino acid terminal sequencing of PSPs

The three parasitism-specific proteins released into the hemolymph of parasitized hosts were transferred to a Sequi-Blot PVDF membrane (Biorad, Hercules, CA) in 10 mM CAPS buffer in 10% methanol (pH 11; CAPS-3-[cyclohexylamino]-1-propanesulfonic acid, Sigma Chemical) at 100 mA for 1.5 h. The membrane was stained with Coomassie rapid blue® R-250 (GelCode, Pierce Company) until bands could be seen, washed twice in Milli-Q water, and destained in 50% methanol, 1% acetic acid in Milli-Q water.

Desired proteins were excised from the membrane, washed in Milli-Q water, and submitted for automated Edman protein sequencing on a Hewlett Packard G1000A Automated Protein Sequencer, using a biphasic, C18 ion-exchange resin-packed column sample support (Edman and Begg, 1967).

Amino terminal sequences obtained by Edman degradation were compared to the Uniprot database using the MPsrch algorithm available at the European Bioinformatics Institute (EMBL-EBI), using the MPsrch_pp program and blosum62 as a matrix (http://www.ebi.ac.uk/MPsrch/) (Altschul et al., 1997). Only matches showing a probability score of $<1.0\times10^{-3}$ were considered significant.

2.6.3. Amino acid composition of PSPs

Proteins were resolved by SDS-PAGE electrophoresis and blotted onto a PVDF membrane as previously described. Proteins bands corresponding to the parasitism-specific proteins were excised from the membrane and hydrolyzed into 0.2 mL thin-wall microtubes, evaporated, reconstituted in borate buffer and analyzed with a Hewlett Packard AminoQuant II System. Derivatization was done using *o*-phthalaldehyde and 9-fluoromethyl-chloroformate, and the derivatized amino acids separated by RP-HPLC and detected by UV absorbance with a diode array detector. Norvaline and sarcosine were used as internal standards to quantify the amino acids. Blank PVDF membranes were used as negative control and albumin was used as a positive control. All samples were injected in duplicate (replicates).

Amino acid data was compared to the Swiss-Prot and TrEMBL databases in order to find proteins with similar amino acid compositions by using the AACompIdent tool available through the Expert Protein Analysis System (ExPASy) proteomics server at the Swiss Institute of Bioinformatics (SIB) (http://ca.expasy.org/) (Wilkins et al., 1998).

2.7. ^{35}S methionine labeling of proteins

The host fat body, teratocytes and the larvae of the developing parasitoid were all tested as possible sources of the parasitism-specific proteins. Dissections were done in anti-coagulant buffer (98 mM NaOH, 0.19 M NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5) (Strand et al., 1997), using parasitized larvae at a stage in which all PSPs were present in the host hemolymph (8–10-d after parasitization).

Parasitized hosts were surface sterilized in 60% ethanol (5 min), washed in sterile water and dissected in anti-coagulant buffer under aseptic conditions. Host fat bodies and developing parasitoid larvae were removed, washed twice in the same buffer and transferred to 150 µl of incubation medium (Table 1) in a 48 well tissue culture plate. Teratocytes were obtained by bleeding parasitized hosts through a severed proleg and collecting the exuding hemolymph into ice-cold anti-coagulant buffer. Samples were then subjected to size-selective filtration using a user-designed filter with a 50 µm cut off membrane to free teratocytes from contaminating host hemocytes. Teratocytes were re-suspended and transferred to a new membrane until hemocytes were no longer observed in the sample, and finally transferred to the incubation medium. Teratocytes were cultured at a density of 20 host equivalents/well.

Incubation was done by adding 10 µCi of trans-labelled ^{35}S methionine and 2.5 µl of antibiotic–antimicotic solution (Gibco BRL) to the incubation medium, and incubated at 26±1 °C for 24 h under constant agitation inside an

Table 1
Composition (mM) of the artificial medium used for the incubation of *H. virescens* fat bodies, teratocytes and larvae of *T. nigriceps*

Component	mM	Component	mM
Amino acids		*Amino acids*	
Alanine	4.1	Valine	4.7
β-alanine	2.0	Threonine	5.1
Arginine	15.4	Tryptophan	4.2
Asparagine	1.1	Tyrosine	10.1
Glutamic acid	3.3		
Glutamine	5.0	*Salts*	
Glycine	4.1	NaCl	20.0
Histidine	8.6	KCl	80.0
Isoleucine	1.7	$CaCl_2$	4.0
Leucine	2.6	$MgCl_2$	15.0
Lysine	12.3	NaH_2PO_4	8.0
Ornithine	0.5		
Phenylalanine	2.2	*Sugars*	
Proline	6.4	Glucose	41.2
Serine	6.6	Trehalose	15.0

incubation chamber saturated with breathing air. Afterwards, proteins were precipitated in cold acetone at −20 °C, re-suspended in TBS buffer, centrifuged and the soluble proteins applied to a discontinuous 7.5% or 10% SDS-PAGE gel slab to evaluate the protein secretory activity of fat body tissue from parasitized hosts, teratocytes and parasitoid larvae. Gels were washed, dried and exposed at −80 °C to a high performance autoradiography film, Hyperfilm MP (Amersham Pharmacia Biotech, UK), using an intensifying screen for at least two weeks.

2.8. Statistics

Statistics were applied on data of carbohydrate and protein composition of the host hemolymph using the statistical program package SigmaStat (SPSS, 1998), and treatments were compared at each sampling interval using the Student's *t*-test ($P<0.05$).

3. Results

3.1. Host regulation—sugars

The quantitative sugar composition of *H. virescens* larvae parasitized by *T. nigriceps* did not differ from that of control larvae, although sugar levels in the hemolymph of parasitized larvae were found to be consistently higher throughout host development (Fig. 1A). Glucose and trehalose were the only sugars constantly identified in the hemolymph of control and parasitized larvae of *H. virescens* in measurable amounts, with sucrose being seldom found. The relative composition of glucose and trehalose was similar in parasitized and control larvae up to host pupation in the control, with the exception that glucose concentration in the parasitized host hemolymph had a sharper decrease than in the control host. Glucose decreased to 20% of the total sugar at day 2 in the hemolymph of parasitized larvae,

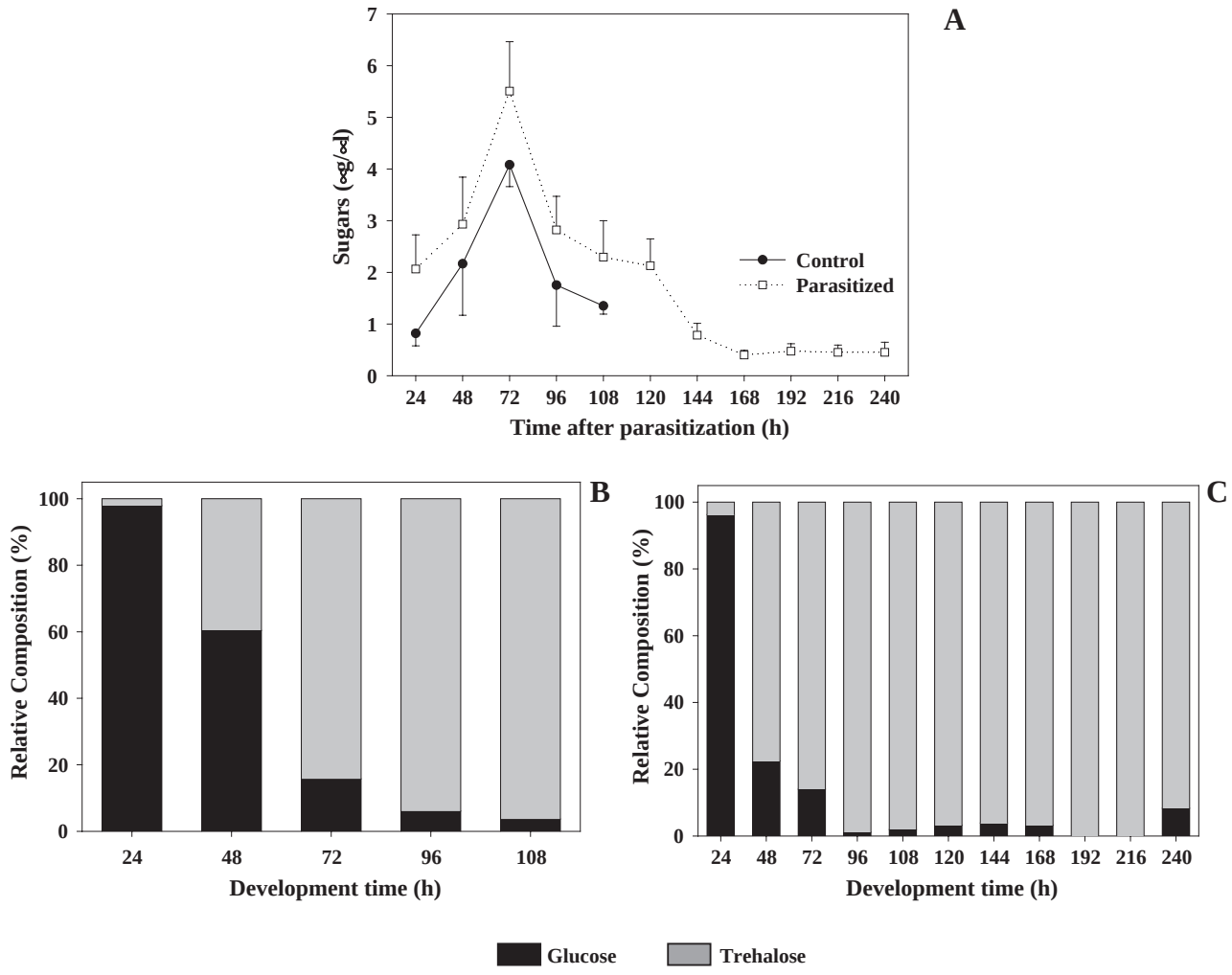

Fig. 1. Quantitative and qualitative carbohydrate composition of 5th instar larva hemolymph of *H. virescens* parasitized by *T. nigriceps* as compared to control hosts (29°±11 °C; 60%±10%; 14L:10D). A—total carbohydrate concentration (μg/μl) of the hemolymph of parasitized (□) and control (●) larvae of *H. virescens*; B—relative composition of trehalose and glucose in the hemolymph during control larvae development; C—relative composition of trehalose and glucose in the hemolymph of parasitized larvae during parasitoid development.

while it still accounted for 60% of the sugar available in the hemolymph of control larvae (Fig. 1B,C).

3.2. Host regulation—proteins

Total protein concentration in the hemolymph of parasitized larvae was similar to that of control larvae up to the wandering stage. The protein concentration in the hemolymph of control larvae fell abruptly shortly before pupation (108 h), with only a slight decrease been observed in parasitized larvae (Fig. 2). Protein concentrations in the hemolymph of parasitized hosts were recovered to the same levels prior to the wandering stage, remaining at ~80 mg/mL throughout the internal larval development of the parasitoid up to egression (240 h), when protein levels were slightly reduced (Fig. 2).

A qualitative analysis of the protein composition of the host hemolymph on a 5%–25% SDS-PAGE did not indicate any difference between the hemolymph of parasitized and control larvae up to shortly before pupation of the control larvae (gels not shown). Two high molecular weight parasitism-specific proteins (PSP1 = 116 kDa and 2 = 114 kDa) were first detected in the hemolymph of parasitized hosts when the control larva was preparing to pupate. A third, 56 kDa parasitism-specific protein (PSP3) was detected in parasitized larvae only at the end of the endoparasitic development of *T. nigriceps* (Fig. 3).

Image densitometry analysis of proteins resolved on an 8% SDS-PAGE slab indicated PSP1 and 2 comprised up to 14% of all the proteins in the hemolymph, while PSP3 was found in much reduced concentration (~5%) (Figs. 3 and 4).

Besides the release of PSPs into the host hemolymph, *T. nigriceps* was found to regulate the levels of three host proteins (Fig. 5). *T. nigriceps* down-regulated an 80 kDa polypeptide (p80), and the protein level achieved up to the

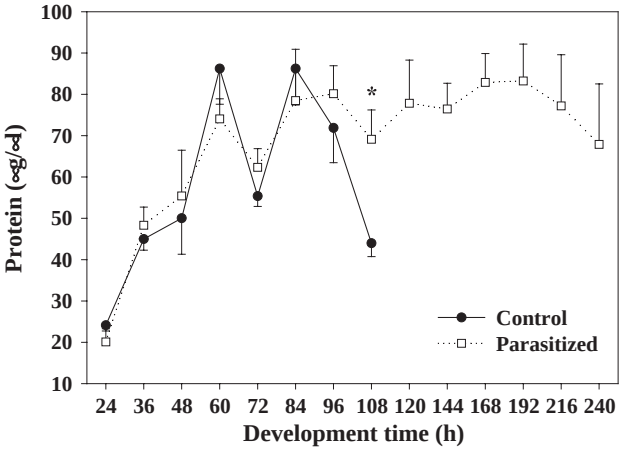

Fig. 2. Total protein concentration of 5th instar larva hemolymph of *H. virescens* parasitized (□) by *T. nigriceps* compared to control (●) hosts (29°±11 °C; 60%±10%; 14L:10D) (* indicates significant differences between treatments at that particular sampling time, *t*-test, $P<0.05$).

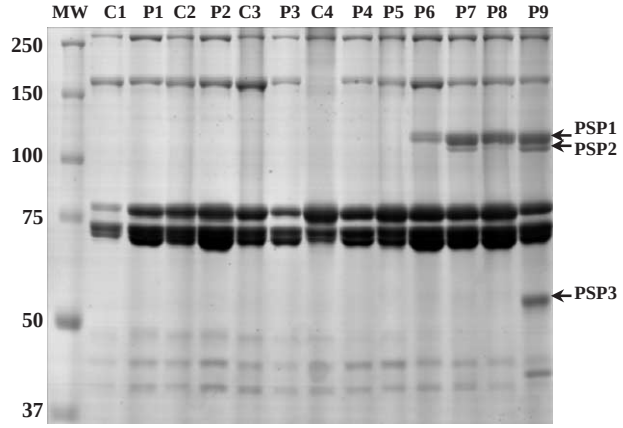

Fig. 3. 8% SDS-PAGE gels showing the protein profile of 5th instar larva hemolymph of *H. virescens* during development of the endoparasitoid *T. nigriceps* as compared to control hosts (29°±11 °C; 60%±10%; 14L:10D). Number designations at the top of each lane represent the intervals samples were collected after parasitization (c=control; p=parasitized). PSP1, PSP2 and PSP3 indicate the parasitism-specific proteins.

time control larva pupated was maintained throughout parasitoid development (Fig. 5B). Two other proteins (173 kDa-p173; 72 kDa-p72), on the other hand, were found in higher levels in the hemolymph of parasitized hosts the day preceding host pupation while sharply decreasing in control larvae (Fig. 5A,D).

3.3. Characterization and identification of the parasitism-specific proteins

Three acidic proteins visualized by IEF electrophoresis in hemolymph samples from parasitized hosts were tentatively identified as the PSP2 (p*I* = 6.3), PSP1 (p*I* = 6.2) and PSP3 (p*I* = 5.8) (Fig. 6), based on the comparison of molecular

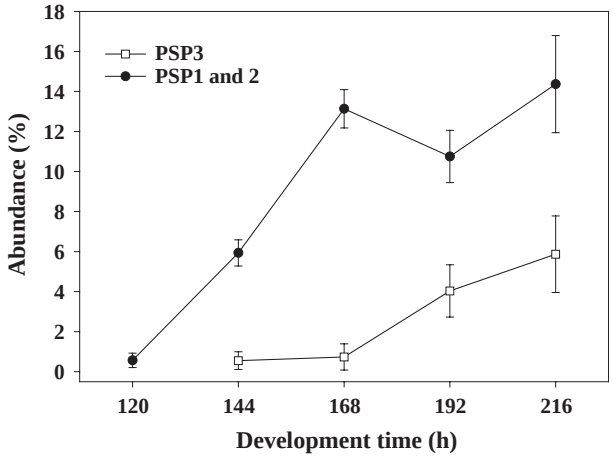

Fig. 4. Densitometric analysis of the SDS-PAGE separated hemolymph proteins showing the abundance (%) of the parasitism-specific proteins (PSP) in the hemolymph of parasitized hosts during development of the endoparasitoid *T. nigriceps*. (PSP1 and PSP2 are represented together because of difficulties in the precise determination of the limits of each band for densitometry).

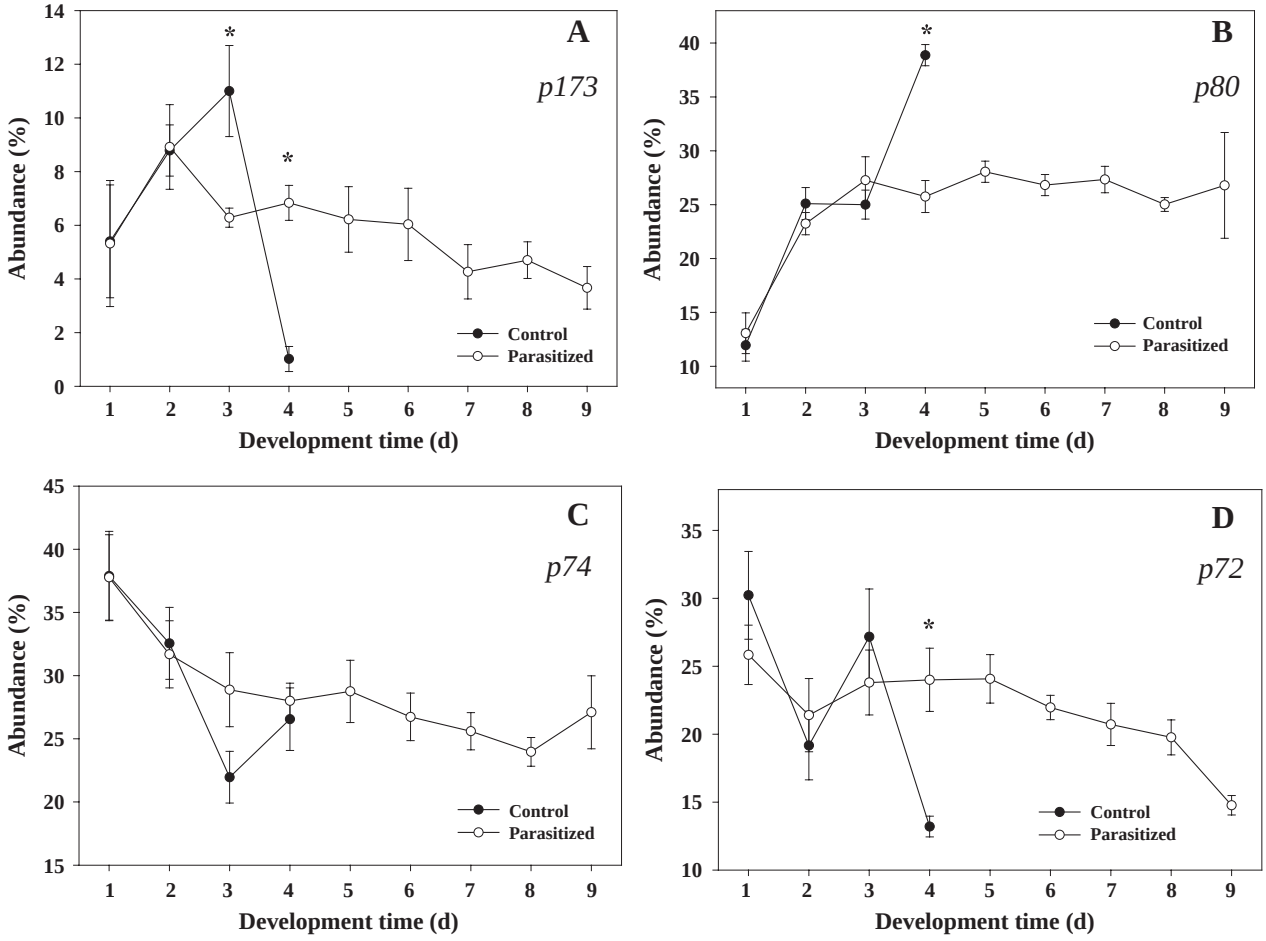

Fig. 5. Densitometric analysis of the 8% SDS-PAGE separated hemolymph proteins showing the abundance (%) of host proteins in the hemolymph of parasitized hosts during development of the endoparasitoid *T. nigriceps* (□) compared to control larvae (●). (p173=putative chromoprotein; p80=monomer of the riboflavin-binding protein; p74=monomer of the arylphorin; p72=monomer of the arylphorin) (* indicates significant differences between treatments at that particular sampling time, *t*-test, $P<0.05$).

weight by SDS-PAGE with that of the identified parasitism-specific proteins.

Results of the amino terminus sequencing of the PSPs produced in the system *H. virescens–T. nigriceps* are shown in Table 2. Database search did not produce any significant matches for PSP1 and PSP2, while the amino terminal sequence obtained for PSP3 yielded significant matches with chitinase and chitinase-like proteins of different insect orders, all at their amino terminal end. The highest score obtained for PSP3 was against a chitinase from the mosquito *Anopheles gambiae* (accession number=Q7Q5I7)($e=8.9e^{-12}$), showing a 62.5% match, with perfect matches for 15 residues and 3 conservative changes. Another relevant match was against an endochitinase precursor from the venom gland of the parasitoid *Chelonus* sp. ($e=8.4e^{-7}$; accession number=Q23737).

Analysis of the amino acid composition of the PSPs produced in the system *H. virescens–T. nigriceps* indicated the 116 (PSP1) and 114 kDa (PSP2) proteins had similar amino acid composition, with glutamic acid+glutamine, aspartic acid+asparagine, isoleucine, leucine and valine making up more than 50% of the total amino acids (Table 3). However, PSP1 had 9-fold more serine than the PSP2 (Table 3). The 56 kDa parasitism-specific protein (PSP3) had aspartic acid+asparagine and glycine as the major amino acids (~25% of the protein composition), with the rest of the protein being composed of about a similar ratio of all other amino acids (Table 3). Once more, web-based database searches using the PSP1 and 2 amino acid composition and physico-chemical properties calculated by SDS-PAGE and IEF electrophoresis did not yield any significant matches.

3.4. Synthesis of parasitism-specific proteins

Proteins with a molecular mass similar to the PSPs released into the host hemolymph during parasitoid development were only found in the media incubated with teratocytes (PSP1=116 kDa; PSP2=114 kDa; PSP3=56 kDa) (PSP3 band is indicated by *, but is very faint and difficult to see in this picture) (Fig. 7). The radiography from gels with proteins released by the parasitoid larvae

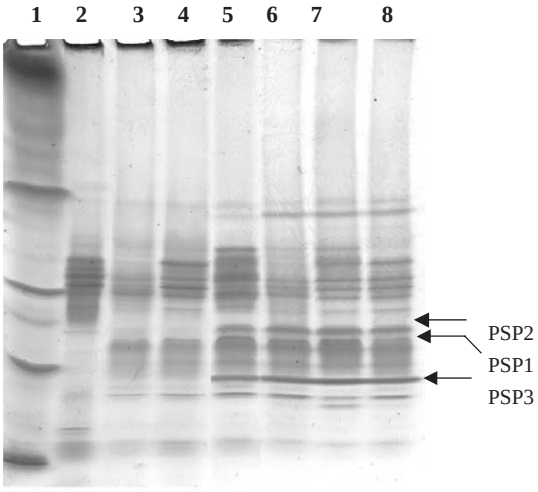

Fig. 6. Five-8 ready IEF gel of the hemolymph of control and parasitized larvae of *H. virescens* indicating the isoelectric point of the parasitism-specific proteins produced in the host parasitized by *T. nigriceps*. Hemolymph samples from control larvae were used to assist in the discrimination of PSPs in the hemolymph from parasitized larvae (1—markers; 2—control hemolymph day 2; 3—control hemolymph day 4; 4 to 9—parasitized hemolymph from days 4 to 9, respectively).

indicated the presence of a protein with a molecular weight a little higher than that of PSP3, and none of the labeled proteins released by the host fat body had a similar mass of the PSPs observed in the hemolymph of parasitized larvae (Fig. 7).

4. Discussion

The physiological mechanisms by which parasitoids overcome the host defense and regulate host development are all ultimately employed to assure the most suitable environment to support parasitoid immature development. Changes in the nutritional quality of the host following parasitization have been reported for a variety of parasitoids, and the implications of altered metabolite levels in host tissues and the consequences of the metabolic redirection and physiological alteration has been discussed for different host and parasitoid associations (Thompson, 1993; Vinson et al., 2001).

Alterations of stored carbohydrates, glycogen and/or trehalose during parasitization in several lepidopteran host species were suggested to occur due to the redirection of exogenous glucose into trehalose and glycogen through regulation of the glycolitic/gluconeogenic pathway (Thompson, 1993; Thompson and Dahlman, 1998), as a way to benefit the developing parasitoid through provision of nutrients. *T. nigriceps* does not regulate the host carbohydrates, but larvae can feed directly on host tissues, which would provide enough carbohydrate and lipids throughout larval development (Dahlman and Vinson, 1975).

Regulation of free amino acid and protein levels in the circulating hemolymph of parasitized hosts has also been suggested as a strategy to increase the overall host suitability to the developing parasitoid (Vinson and Iwantsch, 1980; Edwards and Weaver, 2001). Up- or down-regulation of host proteins may depend on the physiological processes

Table 2
Amino terminal sequence of parasitism-specific proteins identified in the larval hemolymph of *H. virescens* parasitized by *T. nigriceps*

PSP1	X-T-V-N-I-L-Q-L-T-R-I-P-Q-G-K-T-F-V-Y-D-F-Q-A-N-V
PSP2	E-N-I-E-I-R-F-P-V-L-A-S-N-E-N-I-L-V-Y-D-Y-F
PSP3	X-A-I-S-N-Q-E-K-K-I-V-H-Y-F-G-S-W-A-V-Y-R-P-G-R-G

Table 3
Amino acid composition (%) of the parasitism-specific proteins released into the host hemolymph by *T. nigriceps*

Amino acid	PSP1	PSP2	PSP3
Asx	15.7	17.2	13.5
Glx	11.7	11.7	9.6
Ser	4.7	0.5	8.8
His	1.9	3.3	–
Gly	2.0	2.6	14.6
Thr	5.0	3.4	4.2
Ala	4.5	3.7	8.0
Arg	6.1	5.3	4.1
Tyr	5.9	6.2	4.0
Val	7.3	7.9	5.2
Met	0.8	0.4	–
Phe	6.9	6.9	4.1
Ile	8.5	9.6	5.2
Leu	9.9	10.9	8.3
Lys	5.4	6.2	4.7
Pro	4.3	4.2	5.7

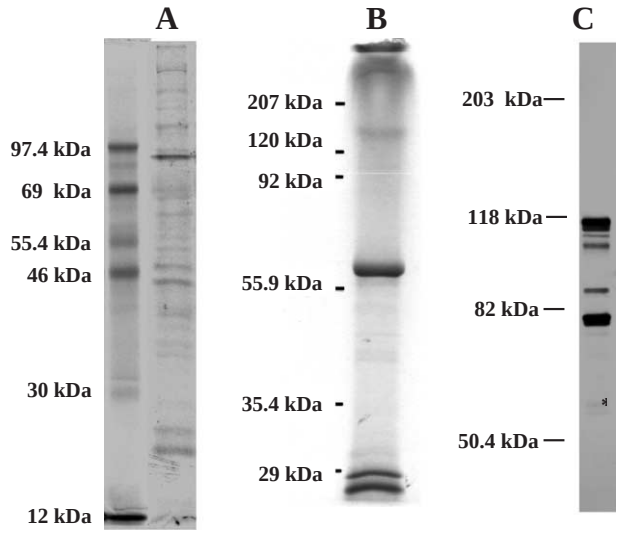

Fig. 7. Autoradiograph of proteins released in the incubation medium by the (A) fat body of 9 d old *H. virescens* parasitized larvae, (B) late 2nd instar parasitoid larvae, (C) and teratocytes of *T. nigriceps* (* indicates the position of the band with a molecular weight similar to PSP3). Larvae and teratocytes were removed from the host 9-d after parasitization. All tissues and parasitoid larvae were incubated in ^{35}S methionine supplemented medium, and proteins were resolved by SDS-PAGE.

they are involved with and the parasitoid life history (Beckage and Kanost, 1993; Dover et al., 1995; Knop-Wright et al., 2001; Vinson et al., 2001). Synthesis and release of proteins derived from parasitoid larvae, associated symbionts or derived tissues with diverse roles also contribute to the changes induced in the protein pattern of the host hemolymph (Harwood et al., 1994; Hochuli and Lanzrein, 2001; Dahlman et al., 2003; Nakamatsu et al., 2002).

Although *T. nigriceps* did not induce an increase in the total protein levels in the hemolymph of parasitized host larvae, it was able to maintain proteins at elevated levels by inhibiting the decrease in protein concentration observed in control larvae preceding pupation (Pennacchio et al., 1994). The maintenance of proteins, which are taken up by the fat body at the onset of the metamorphosis, in the hemolymph of parasitized larvae points to mechanisms of regulation of the protein uptake process (Haunerland, 1996). Two of the host proteins regulated during parasitism showed reduced titers as the host approached pupation in the control larva. The molecular size and dynamics of the p72 and p80 in the hemolymph during host development and their reaction with antisera raised against the host storage proteins (data not shown), led us to conclude that they are monomers of the small arylphorin subunit and riboflavin-binding protein, respectively (Leclerc and Miller, 1990). The molecular weight, abundance and time of appearance and clearance of the p173 from the host hemolymph are all indicators that it may correspond to the host chromoprotein (Leclerc and Miller, 1990).

It has been demonstrated in *H. virescens* that while p80 remains in the hemolymph as the insect approaches pupation, monomers of the arylphorin (p74 and p72) and the chromoprotein (p173) are taken up by the fat body (Leclerc and Miller, 1990). These peptides are by far the most abundant proteins in the hemolymph of last instar larvae of *H. virescens*, comprising more than 80% of the total hemolymph proteins (Leclerc and Miller, 1990).

In endoparasitoids, both teratocytes and polydnavirus have been implicated in the regulation of storage proteins of parasitized hosts (Pennacchio et al., 1994; Shelby and Webb, 1997; Schepers et al., 1998; Dahlman et al., 2003). However, in all these cases, levels of storage proteins were reduced in the parasitized larvae. Teratocytes, in particular, were shown to inhibit synthesis of the large monomer of the host arylphorin in the system *H. virescens–M. croceipes* (Schepers et al., 1998), the only monomer (p74) of the host storage proteins that was not affected by *T. nigriceps*.

Regulation of host to the host storage proteins is certainly a way parasitoids have to control host energy allocation and development, but it could also have a nutritional role for parasitoid development (Vinson et al., 2001; Knop-Wright et al., 2001; Dahlman et al., 2003). The mechanisms by which regulation occur are certainly influenced by the parasitoid developmental biology and strategies of nutrient utilization employed by a particular species. Host metabolic pathways can be affected resulting in the redirection of primary nutrients that would be used in the synthesis of molecules by the developing parasitoid or derived tissues (Rahbe et al., 2002), or be directly absorbed or metabolized by the developing parasitoid (Eguileor et al., 2001; Knop-Wright et al., 2001). In addition, down-regulation of host proteins at the translation level has been suggested as a mechanism to improve host suitability by reducing the host metabolic costs, since 83% of the cellular energy is allocated to protein synthesis (Pannevis and Houlihan, 1992; Dahlman et al., 2003).

However, the mechanism of regulation of host proteins by *T. nigriceps* is unknown. Expression and/or abundance of several insect proteins are hormone regulated or dependent on the insect feeding activity or on the insect developmental stage (Riddiford and Hice, 1985; Terwilliger, 1999). Both host hexamerins regulated during development of the parasitoid *T. nigriceps* are controlled by either ecdysone or juvenile hormone (JH). Riboflavin-binding proteins, which are orthologous to the JH-suppressible proteins, are down-regulated in the presence of JH (Burmester, 1999; Godlewski et al., 2003). The drop in the levels of this protein (p80) in the hemolymph of parasitized hosts at the time control larvae approach pupation correlate with the higher levels of JH in parasitized hosts (Li et al., 2003).

Synthesis of arylphorin is under a different control mechanism in which host feeding activity and ecdysone titers control mRNA synthesis (Riddiford and Hice, 1985). Arylphorin transcripts are reduced during the molt period and almost depleted after the wandering stage ecdysone titers rise (Riddiford and Hice, 1985). The reduced feeding activity and ecdysone titers of hosts parasitized by *T. nigriceps* indicate regulation of the arylphorin is at the transcriptional level (Guillot and Vinson, 1973; Pennacchio et al., 2001). Therefore, maintenance of arylphorin in the hemolymph of parasitized hosts also indicates that the uptake process of this protein is also under parasitoid control. Uptake could either be regulated by inducing structural modifications in the protein by affecting post-translational pathways or by shutting down the receptor-mediated mechanism of protein recognition and uptake in the target tissue (fat body) (unpublished data). If host suitability is to be improved by minimizing the energetic costs associated with protein synthesis (Dahlman et al., 2003), regulation of the uptake of proteins might be another mechanism to provide steady concentration of proteins to sustain parasitoid larval development with a lower energetic investment by the host.

Proteins with molecular weight similar to the PSPs identified in the hemolymph of parasitized hosts were only released by teratocytes. The tentative identification of teratocytes as the releasing tissue of PSPs was corroborated after amplification and sequencing of a transcript using mRNA from teratocytes in a RT-PCR reaction for PSP3. The amino acid sequence of the amplified transcript matched that obtained by amino terminal sequence of the protein,

confirming PSP3 was a teratocyte product (unpublished data).

Teratocytes are known to play a diverse role in the many systems (Dahlman and Vinson, 1993).The appearance of the two unidentified PSPs likely produced by teratocytes of *T. nigriceps* (PSP1 and PSP2) coincided with the period host pupation is halted, and their increase in titer during parasitism could suggest their involvement in processes regulating the arrestment of host development. Teratocytes of *T. nigriceps* were earlier demonstrated to affect host development by regulating the host ecdysteroid metabolism (Pennacchio et al., 2001). However, their appearance is also timed with the molt of the first to second instar parasitoid larva, a physiological event that has been shown to be synchronized with the host development and dependent on an increase in protein concentration (Pennacchio et al., 1993). Therefore, these proteins could also play a nutritional role in parasitoid development, as demonstrated for teratocyte-produced proteins in another system (Kadono-Okuda et al., 1998).

The putative chitinase (PSP3) appeared just before the egression of the parasitoid larva from the host, and could be involved in the facilitation of the parasitoid larva egression by aiding on the digestion of the host cuticle, since the parasitoid larva lacks an elaborated mandibular apparatus (Lewis and Vinson, 1968).

In conclusion, *T. nigriceps* was shown to regulate the host protein levels and the abundance of host-specific proteins during parasitism that could have a nutritional role in supporting parasitoid development. Teratocytes are probably the source of detectable PSPs into the host hemolymph. Two of these proteins (PSP1 and PSP2) showed no similarities with known proteins in the database search using their amino terminal sequences or amino acid composition and physico-chemical properties as entries, while PSP3 was identified as a putative chitinase. PSP3 has been assigned a putative function of aiding parasitoid larva egression by digesting the host cuticle. Isolation and sequencing of the transcripts of the PSPs produced by teratocytes of *T. nigriceps* and further structural analysis of these proteins will allow for their characterization and shed light on their role in this host-parasitoid association.

Acknowledgements

We would like to thank the Protein Chemistry Laboratory, Texas A and M University, for providing the amino terminal sequence and amino acid analysis for the PSPs produced by teratocytes of *Toxoneuron nigriceps*.

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